

**Applicant:**

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**Title**

Novel virus encoded chemokines determine the tissue tropism of human cytomegalovirus (HCMV).

## Description

The previous patent application PCT/EP02/01867 (WO 02/066629) describes the cloning of FIX-BAC and assigned the genetic determinants of leukocyte and endothelial cell tropism of HCMV to the UL132-UL128 genetic locus of HCMV.

Figure 1

### Cloning of the endothelial cell tropic and leukotropic clinical isolate VR1814 as FIX-BAC

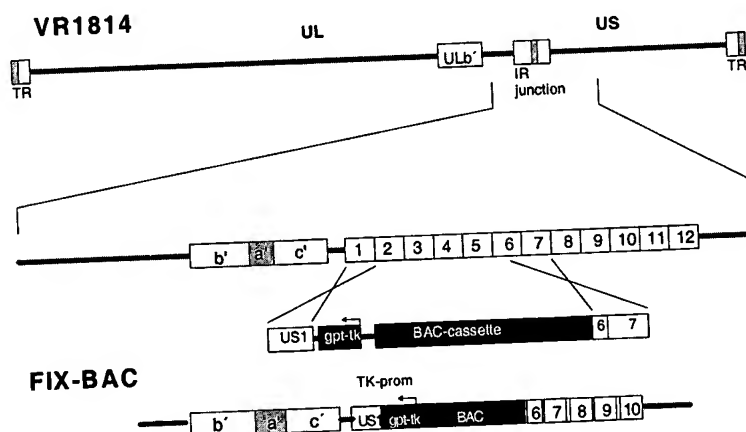


Fig. 1 Construction of FIX-BAC. A gpt-tk-BAC cassette was introduced into the US2-6 region of the parental virus VR1814 as described in patent application PCT/EP02/01867 (WO 02/066629).

In the current patent application specific virus mutants targeting individual genes within the UL131-128 region as well as at the borders of UL131-128 were constructed using a sited-directed PCR-based approach in *E. coli*. Recombination functions of bacteriophage  $\lambda$  ( $\alpha/\beta/\gamma$ ) were transiently provided on a plasmid in *E. coli* DH10B containing at least one copy of the FIX-BAC genome. The recombination fragment was generated by PCR over a kanamycin resistance marker (pACYC177, NEB Biolabs).

The following primers were used:

**RVFIXΔUL130-132** (P-132-for: 5'-AAA CCA CGT CCT CGT CAC ACG TCG TTC GCG GAC ATA GCA AGA AAT CCA CGT CGC CAC ATC TCG AGA CGA TTT ATT CAA CAA AGC CAC G-3'; P-130-rev: 5'-AAC GGC GTC AGG TCT TTG GGA CTC ATG ACG CGC GGT TTT CAA AAT TCC CTG CGC GCG CGA CGG GCG CCA GTG TTA CAA CCA ATT AAC C-3'); **RVFIXΔUL130** (P- 130-for: 5'- GCG CCA CAC GCC CGG AGC CTC GAG TTC AGC GTG CGG CTC TTT GCC AAC TAG CCT GCG TCA CGG CGA TTT ATT CAA CAA AGC-3'; P-130-rev: 5'-AAC GGC GTC AGG TCT TTG GGA CTC ATG ACG CGC GGT TTT CAA AAT TCC CTG CGC GCG CGA CGG GCG CCA GTG TTA CAA CCA ATT AAC C-3'); **RVFIXΔ128K** (P-128-for-kons: 5'-TGC GTT CTG TGG TGC GTC TGG ATC TGT CTC TCG ACG TTT CTG ATA GCC ATG TTC CAT CGA CGA TTT ATT CAA CAA AGC CAC G-3', P-128-kons2: 5'- CGG CAC ACA TCC AGC CGT TTG TGT TTC TTA ACG CTC TCC AGG TAC TGA TCC AGG CCC ACG GCC AGT GTT ACA ACC AAT TAA-3') **RVFIXΔUL127** (P-127-for: 5'-TTG AGA TTT CTG TCG CCG ACT AAA TTC ATG TCG CGC GAT AGT GGT GTT TAT CGC CGA TAG CGA TTT ATT CAA CAA AGC CAC-G3', P-127-rev: 5'- AAT ATT GAT TTA CGC TAT ATA ACC AAT GAC TAA TAT GGC TAA TGG CCA ATA TTG ATG CAA GCC AGT GTT ACA ACC AAT TAA-3'); **RVFIXΔUL148** (P-148-for: 5'- GAC TAT GTG CAT GTT CGG CTA CTG AGC TAC CGA GGC GAC CCC CTG GTC TTC AAG CAC ACT CGA TTT ATT CAA CAA AGC CAC-3', P-148-rev: 5'- CAC CAG GTA GGT TAT CAA AAC GCG AGC CCA TAT CGC CGC CAT CAT TGT AAT CAG CAA TGT GCC AGT GTT ACA ACC AAT TAA-3'); **RVFIXΔUL132K** (P-132-forK: 5'- ACG TCC TCG TCA CAC GTC GTT CGC GGA CAT AGC AAG AAA TTC ACG TCG CCA CGT CTC GAG ACG ATT TAT TCA ACA AAG CCA-3', P-132-revk: 5'-AAG GTT CTT CCA TTT CCG AGG CGG TCA

GTT CAT CGT ACA CCG AGA CGT AGT ACC TGA TGG GGC CAG TGT TAC AAC  
CAA TTA ACC-3'); **RVFIXΔUL132-128** (P-131-for: 5'-TGT CTT TCG GTT CCA ACT  
CTT TCC CCG CCC CAT CAC CTC GCC TGT ACT ATG TGT CGA TTT ATT CAA  
CAA AGC CAC G- 3'; P-128-rev: 5'-TCG CGC GAC ATG AAT TTA GTC GGC GAC  
AGA AAT CTC GAA ACG CGT ATT TCG GAC AAA CAC ACA TGC CAG TGT TAC  
AAC CAA TTA ACC-3'); **RVFIXΔUL133-148** (P-133-for: 5'-CGC TGT AGG GAT AAA  
TAG TGC GAT GGC GTT TGT GGG AGA ACG CAG TAG CGA TGG GTT GCG ACG  
TGC ACC GAT TTA TTC AAC AAA GCC ACG-3', P-148-rev: 5'- CAC CAG GTA  
GGT TAT CAA AAC GCG AGC CCA TAT CGC CGC CAT CAT TGT AAT CAG CAA  
TGT GCC AGT GTT ACA ACC AAT TAA-3'); **RVFIXΔUL131K** (P-131-fork: 5'-CAG  
TCT GCA ACA TGC GGC TGT GCT GGG TGT GGC TGT CTG TTT GTC TGT GCG  
CCG TGG TGC CGA TTT ATT CAA CAA AGC CAC-3', P-131-rev: 5'-GCT AGT  
TGG CAA AGA GCC GCA CGC TGA ACT CGA GGC TCC GGG CGT GTG GCG  
GCC AGT GTT ACA ACC AAT TAA CC-3'); **RVFIXΔ146-147** (P-UL146-for: 5'-GAT  
TTT CCG GGA ATA CCG GAT ATT ACG AAT TAC TGG TAG TGA CGT AGA TAA  
TAA AAT TAT ACG ATT TAT TCA ACA AAG CCA CG-3', P-UL147-rev: 5'-CAC  
CAA AGC CGT TAG CGT GCC CAG AGC TAC CGC ACG GTA AAA TAG GGA  
CAT GAG CCA GTG TTA CAA CCA ATT AAC C-3').

Figure 2

<b>RVFIX virus mutants</b>	<b>Deletion according to (Chee, Bankier et al., 1990)</b>
RVFIXΔULb'	nt 175662-189347
RVFIXΔUL132-128	nt 174857-176865
RVFIXΔUL133-148	nt 177896-189347
RVFIXΔUL132-130	nt 175662-177743
RVFIXΔUL131K	nt 176377-176776
RVFIXΔUL130	nt 175662-176314
RVFIXΔUL128K	nt 174953-175387
RVFIXΔUL132K	nt 177076-177752
RVFIXΔUL148	nt 177896-178644
RVFIXΔUL146-147	nt 179069-179966
RVFIXΔUL127	nt 174467-174770

Fig. 2 List of RVFIX virus mutants and determination of the mutation (kanamycin cassette insertion) according to annotation by {Chee, Bankier, et al. 1990 269 /id}

Figure 3 shows an agarose gel and Southern Blot of RVFIX and RVFIX mutant viruses.

Figure 3

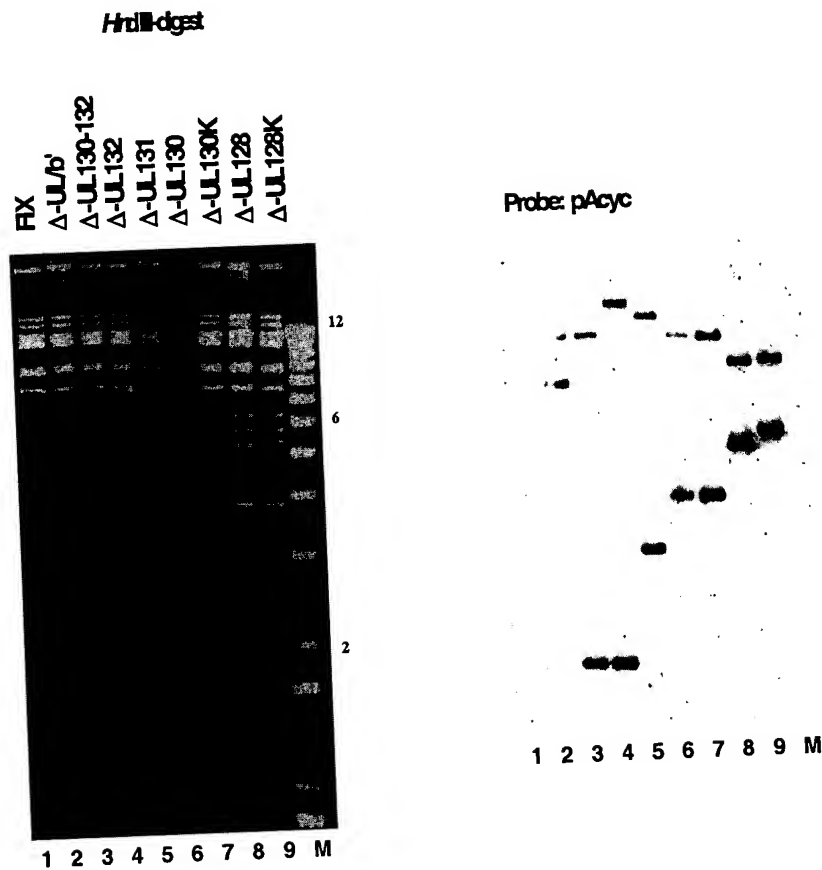


Fig.3 DNA derived from individually grown FIX-BAC clones (lane 1) or FIX-BAC mutant clones  $\Delta$ ULb',  $\Delta$ UL130-132,  $\Delta$ UL132,  $\Delta$ UL131,  $\Delta$ UL130,  $\Delta$ UL130K,  $\Delta$ UL128 and  $\Delta$ UL128K (lanes 2-9) was digested with *Hind*III and run on a 0.5% agarose gel. The Southern Blot was probed with the kanamycin specific probe pAcyc. The generation and testing of the mutants is described in the text. M: molecular weight marker 1 kb ladder.

Tabel 1 summarizes the phenotypical testing of RVFIX virus mutants.

**Table 1: Leukocyte, Monocyte and HUVEC tropism**

	PMNL-Tropism	MO-Tropism	HUVEC-Tropism
RVFIX WT	positive	positive	HUVEC+
RVFIX $\Delta$ UL130	negative	negative	HUVEC-
RVFIX $\Delta$ UL131	negative	negative	HUVEC-
RVFIX $\Delta$ UL132	positive	positive	HUVEC+
RVFIX $\Delta$ UL128	negative	negative	HUVEC-
RVFIX $\Delta$ UL131-128	negative	negative	HUVEC-
RVFIX $\Delta$ UL127	positive	positive	HUVEC+
RVFIX $\Delta$ UL148	positive	positive	HUVEC+
RVFIX $\Delta$ UL133-148	weak pos.	positive	HUVEC+
RVFIX $\Delta$ UL146-147	weak pos.	positive	HUVEC+

PMNL: Peripheral blood mononuclear leukocytes  
MO: Monocytes  
HUVEC: Human umbilical vein endothelial cells

Only mutants with a deletion of either individual genes UL131, UL130, UL128 or the entire UL131-128 locus lost tropism for both endothelial cells and leukocytes. When genes next to UL131-128 were deleted (UL132, UL127 or UL148) the virus mutants were perfectly capable of infecting endothelial cells and leukocytes confirming the UL131-128 locus as the genetic determinant of endothelial cell and leukocyte

tropism. When a deletion between UL133-148 was introduced into the RVFIX genome the resulting virus RVFIX $\Delta$ UL133-148 retained the capability to infect endothelial cells and leukocytes, however a predominant monocyte tropism was observed. Since it is known from literature (Penfold, Dairaghi et al., 1999; Mocarski, Jr., 2002) that UL146 is a functional CxC chemokine, the predominant monocyte tropism of the virus mutant RVFIX $\Delta$ UL133-148 was attributed to the loss of UL146. In order to confirm this notion, a virus mutant was constructed which UL146-UL147 was deleted. The virus mutant RVFIX $\Delta$ 146-147 confirmed the RVFIX $\Delta$ UL133-148 phenotype.

The role of leukocyte attraction in cytomegalovirus infection was recently underscored by the finding that, in MCMV, two differentially spliced CC chemokine homologs (Fleming, Davis-Poynter et al., 1999; MacDonald, Li et al., 1997; MacDonald, Burney et al., 1999) encoded by m131-129 (MCK-1 and MCK-2) are important determinants of virus dissemination (MacDonald, Li, & Virgin, 1997; MacDonald, Burney, Resnick, & Virgin, IV, 1999; Saederup, Lin et al., 1999; Saederup, Aguirre et al., 2001), acting as proinflammatory signals that recruit leukocytes to the site of infection to increase virus spreading. The relevance of chemoattraction is highlighted in this study by the phenotype of mutants RV $\Delta$ UL146-147 and RV $\Delta$ UL133-148, both bearing a functional UL131-128 locus while lacking the viral CXC chemokine genes UL146-147. UL146 product is a potent attractor and activator of human PMNL *in vitro* (Penfold, Dairaghi, Duke, Saederup, Mocarski, Kemble, & Schall, 1999). The mentioned virus mutants RV $\Delta$ UL146-147 and RV $\Delta$ UL133-148 share a full endothelial cell and monocyte tropism, but are only inefficiently transmitted to PMNL (polymorphonuclear leukocytes). These results suggest that genes within UL131-128 are sufficient both for local endothelial spread

and for attraction of (and virus passage to) monocytes, while the additional chemotactic factors encoded by UL146-147 enhance virus dissemination by attracting PMNL and favouring their infection. The chemotactic activity of each individual gene product of the UL131-128 locus, as well as the cooperation with other viral or cellular gene signalling molecules, remain to be further elucidated.

As described in patent application PCT/EP02/01867 (WO 02/066629) 5' and 3' RACE analyses had led to the identification of novel viral transcripts running through the genetic locus UL131-UL128 with a start codon at the beginning of UL131 and a stop codon and poly A signal at the end of UL128. The current patent application provides a more in depth analysis of the newly identified transcripts on the transcriptional and translational level. Figure 4 A-C shows a sequence comparison of the newly identified RACE clones 95-3, 95-8 and 95-11 to FIX-BAC genomic sequence. Figure 5 shows the entire UL131-128 genetic locus and predicted individual genes.

**Transcript analysis was performed by rapid amplification of cDNA ends (RACE).** First strand cDNA synthesis and rapid amplification of cDNA ends (RACE) was performed using the SMART™ RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. RACE products were cloned into the pT-Adv vector (Clontech) using the AdvanTage™ PCR cloning kit for analysis and sequencing. For rapid amplification of cDNA ends (RACE) from the 5' RACE cDNA sample the following primers were used: primer 57-GSP1: 5'- CGG CAC ACA TCC AGC CGT TTG TGT TTC TTA 3'; primer 72-GSP1: 5'-TAA CGC TCT CCA GGT ACT GAT CCA GGC CCA -3'; primer 73-GSP1: 5'-TCG TCA GTT TGT TGT GTA CGA CCT GGC GTG-3'; primer 74-GSP1: 5'-TAT TGG CCT CGG TGA ACG TCA



ATC GCA CCT -3'. For rapid amplification of cDNA ends from the 3' RACE cDNA sample the following primers were used: primer 56-GSP2: 5'-TGT GTC GGG TGT GGC TGT CTG TTT GTC TGT-3'; primer 75-GSP2: 5'-TCT GCT TCG TCA CCA CTT TCA CTG CCT GCT-3'; primer 76-GSP2: 5'-CGC AGA AGA ATG TTG CGA ATT CAT AAA CGT-3'; primer 77-GSP2: 5'-GCT GCG GTG TCC GGA CGG CGA AGT CTG CTA-3'; primer 78-GSP2: 5'-CCA GCT GGC AGA TTC CCA AAC TAA TGA AAG-3'; primer 93-GSP2: 5'-CTT TCG GTT CCA ACT CTT TCC CCG CCC CAT-3'; primer 94-GSP2: 5'-CAC CTC GCC TAT ACT ATG TGT ATG ATG TCT-3'; primer 95-GSP2: 5'-CTC TCT TTC TCA GTC TGC AAC ATG CGG CTG-3'; primer 96-GSP2: 5'-GTT GTC CAA GCC GTC GCT CGC ATC GTA GTG-3'; primer 97-GSP2: 5'-CAT AAT AAA GCT CTC TTT CTC AGT CTG CAA-3'; primer 98-GSP2: 5'-TAT GAT GTC TCA TAA TAA AGC TCT CTT TCT-3'.

#### **Identification of novel tropism determining transcripts**

The novel and previously unrecognized transcripts running through the entire UL131-128 region with a predicted UL131 start codon (nt 176825-176823 according to (Chee, Bankier, Beck, Bohni, Brown, Cerny, Horsnell, Hutchison, III, Kouzarides, Martignetti, & ., 1990), and a UL128 stop codon (nt 174865-174863) were identified. The newly identified transcripts show a splicing event between UL128x2 and UL128x3 (nt 175201-175081), either exclusively or in conjunction with splicing between UL131x1 and UL131x2 (nt 176589-176480). An additional splicing event between UL128x1 and UL128x2 (nt 175459-175335) was observed in several clones.

3' RACE analysis consistently identified a single polyA stretch  $14 \pm 1$  nucleotides 3' to the canonical AATAAA polyA signal immediately downstream of the UL128 stop codon.

As depicted in Fig. 6 and translation of RACE clone 95-3 (Fig. 9) and RACE clone 95-8 (Fig. 10) shows predicted proteins with a CxC motif (red) which is characteristic for CxC chemokines. RACE clone 95-3 encodes a 129 aa protein designated HCK-1 (pUL131; about 15 kDA) and RACE clone 95-8 encodes a 79 aa protein designated HCK-2 (pUL131 x 1; about 9 kDA). Both proteins show a number of N-linked glycosylation sites.

Both newly identified transcripts (RACE clone 95-3 and RACE clone 95-8) have a splice between UL128x1 and UL128x2. RACE clone 95-3 has an additional splice between UL131x1 and UL131x2 which is absent in RACE clone 95-8. Sequence comparison between the RACE clones, FIX-BAC and AD169 genomic sequences revealed that a stretch of 7 x nt A (blue) in UL131 of FIX-BAC and the RACE clones 95-3 and 95-8 is extended to a stretch of 8 x nt A (blue) in AD169 genomic sequence. Translation of the RACE clones 95-3 (Fig. 9) and 95-8 (Fig. 10) showed an open reading frame (ORF) with a conserved CxC C C chemokine motif (red) in FIX-BAC which is destroyed by the extension of 7 x nt A to 8 x nt A in the fibroblast adapted laboratory strain AD169. In RACE clone 95-3 the splice between UL131x1 and UL131x2 removes the stop codon at the end of UL131x1 and codes for a putative 129 aa CXC chemokine like protein (designated HCK-1; Human cytomegalovirus chemokine like protein), whereas in RACE clone 95-8 the stop codon at the end of UL131x1 is used to truncate the CXC chemokine like protein HCK-2 to 79 aa.

Putative CC chemokine like proteins designated HCK-3 and HCK-4 are encoded by the predicted UL128 ORF (Fig. 7). However, RACE analyses have not yet fully confirmed the 5' start of these transcripts. HCK-3 (pUL128x1) (Fig. 12) is a

59 aa protein (about 7 kDA) and HCK-4 (pUL128) (Fig. 13) is a 171 aa protein (about 20 kDA). Splicing events between UL128x1, UL128x2 and UL128x3 fuse the three UL128 exons to form the 171 aa protein HCK-4. In case of HCK-3 the splice between UL128x1 and UL128x2 is absent and thus the TGA stop codon at the end of UL128x1 truncates the protein HCK-3 to 59 aa.

RACE clone 95-11 (Fig. 11) confirms that UL128 is composed of three exons.

Interestingly, in RACE clone 95-11 the stretch of 7 x nt A (present in RACE clones 95-3 and 95-8) is extended to a stretch of 9 x nt A. This extension from 7x to 9 x nt A destroys the CxC motif in RACE clone 95-11 and takes the predicted HCK-1 and HCK-2 proteins out of frame. It can be speculated that by increasing or decreasing the numbers of nt (for example nt A, nt T, nt G or nt C) in a given transcript, HCMV has found a way of transcriptional regulation of tissue tropism. It can be pictured that in a given cell type (for example fibroblasts) predominantly one transcript (for example 95-11) is synthesized over others (for example 95-8 or 95-3), thereby introducing a frame shift in an open reading frame (for example HCK-1 and HCK-2) whose product is necessary for tissue specific infection. It can also be pictured that in other cells for example endothelial cells, monocytes, leukocytes, dendritic cells or progenitor cells predominantly those transcripts are made (for example 95-3, 95-8, 128A and 128B) whose protein products (for example HCK-1, HCK-2, HCK-3 and HCK-4) encode viral chemokines and microfusion inducing factors (for example UL130, HCK-5) (Fig. 14).

Closer analysis of the newly encoded proteins HCK-1 and HCK-2 shows that they have N-linked glycosylation sites. It can be speculated that HCK-1 may be membrane bound and is trafficking through the endoplasmatic reticulum. This membrane bound HCK-1 could be of crucial importance for inducing the microfusion

event (in conjunction with pUL130, HCK-5) between endothelial cells and leukocytes, monocytes, macrophages and dendritic cells or possibly other cell types. It can be assumed that HCK-2 may be a soluble chemokine which would be necessary to attract leukocytes (in conjunction with the CxC chemokines UL146 and UL147) to the site of infection. Thus HCK-1 and HCK-2 are major pathogenicity factors for virus dissemination *in vivo* and *in vitro*. It can further be assumed that the newly identified CC chemokines HCK-3 and HCK-4 may attract monocytes, macrophages, dendritic cells and possibly hematopoietic progenitor cells or stem cells to the site of infection and that concomitantly the infectious virus is spread via microfusion possibly by the use of HCK-1 and HCK-5 protein products. It can be pictured that chemokine receptors interact with the HCK-1, HCK-2, HCK-3, HCK-4 and HCK-5 protein and that the microfusion event occurs via receptor internalisation. Adhesion molecules which are upregulated by HCMV infection may provide assistance in the attachment, recruitment and microfusion process.

### **Transcript analyses by Northern Blot**

Northern blots from fibroblasts infected with RVFIX (Fig. 8), when hybridized with a UL131-128 specific probe, showed an upper (2.0-1.8 kb) band and a lower (0.8-0.7 kb) band. The 0.8-0.7 kb band can be interpreted as a UL128-specific transcript, the 2.0-1.8 kb band as the long UL131-128 encompassing transcript. Independent promoters may drive each transcript, one predicted upstream of the UL131 gene and the other within the UL131-130 region. The lack of a UL130 specific transcript suggests that the UL130 protein may be translated from the polycistronic 2.0-1.8 kb mRNAs through either translational reinitiation or an IRES-like mechanism.

Northern analyses of RVFIX $\Delta$ UL130 or RVFIX $\Delta$ UL131K infected fibroblasts shows that following the kan<sup>R</sup> cassette insertion into either UL131 or UL130 the stability of all transcripts of the UL131-128 region was altered (2.0-1.8 kb transcript was absent and 0.8-0.7 kb transcript was strongly diminished), whereas kan<sup>R</sup> insertion into RVFIX $\Delta$ UL128 shifted both transcripts (Fig.8). In Toledo infected cells both mRNA bands are missing as a consequence of truncation of UL128 and dislocation of the polyA signal (Fig. 8). Taken together, these data suggest that a targeted deletion of individual genes within UL131 to UL128 affects the stability of transcripts of the entire locus. Functionally, the lack of endothelial cell and leukocyte tropism observed in Toledo may correlate with the failure to express all UL131-128 transcripts. In contrast, point mutations in the UL131-128 region of the laboratory strain AD169 as compared to RVFIX neither affect mRNA mobility nor stability. However, in AD169 a stretch of 7 x nt A in RVFIX is extended to 8 x nt A in laboratory strain AD169. As a result, the ORF of pUL131 (HCK-1) and pUL131x1 (HCK-2) in AD169 is frameshifted.

By construction of individual virus mutants which delete individual genes UL131, UL130, UL128 it could be demonstrated that HCMV loses its tropism for monocytes, leukocytes and endothelial cells when the genetic region coding for the viral proteins HCK-1, HCK-2, HCK-3, HCK-4 or HCK-5 is removed from the virus genome. It is reasonable to assume that infection of cell types such as dendritic cells, macrophages, progenitor cells, B- and T- lymphocytes, hematopoietic stem cells may occur in the same fashion and that the UL131-128 locus of HCMV and its encoded protein products described are indispensable for the infection process *in vivo* and *in vitro*.

**Rescue of leukotropism and endothelial cell tropism by ectopic reinsertion of the UL131-128 region of FIX-BAC into the laboratory strain AD169.**

In order to show that the UL131-128 region of FIX-BAC is sufficient to rescue tissue tropism of a an endothelial cell tropism and leukotropism incompetent strain, the UL131-128 genetic locus of FIX-BAC was cloned into the vector pORI6K-zeo next to an FRT site. In AD169-BAC the UL40 region was deleted and replaced with a kanamycin cassette flanked by FRT sites (from plasmid pcp015). Subsequently the kanamycin resistance marker was removed by flip recombinase (provided by plasmid pcp20) in *E. coli*. Ectopic insertion of the UL131-128 region from FIX-BAC (cloned into pORI6K-zeo) into AD169 $\Delta$ UL40FRT-BAC was achieved by flip recombinase in *E. coli* and selection for kan<sup>R</sup> and zeo<sup>R</sup> resistant clones. The reconstituted virus RVAD169 $\Delta$ UL40+UL131-128 could infect endothelial cells and leukocytes. However, the leukocyte tropism was predominantly restricted to monocytes, as expected in a virus which lacks the CxC chemokine coding genes UL146 and UL147.

The genes UL131, UL130, UL128 and the protein products with novel structure encoded (namely HCK-1, HCK-2, HCK-3, HCK-4 and HCK-5) are of fundamental importance for infection, dissemination and spread of HCMV in the human body. It can be pictured that these genes and proteins are key players in disease conditions such as vascular disease and atherosclerosis development. They are targets for drug design (small molecules, anti-sense RNA, siRNAs), anti-viral chemotherapy, vaccine development and gene therapy against HCMV and other virus induced diseases (for example HIV), as well as diseases such as cancer, autoimmune disorders and atherosclerosis. Since viruses like HCMV co-evolved with the host during human evolution it, is reasonable to assume that the newly described CxC and CC chemokines of novel structure may have as yet unidentified chemokine homologues in humans (for example secreted by human immune cells).

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